REMARKS

As a preliminary, Applicants and Applicants' representative thank the Examiner and her Supervisor for the personal interview held on May 23, 2005.

By the present amendment, claims 7-9 have been canceled and new claims 12-34 have been added. Support for new claims 12-34 is found in original claims 7-9 and page 11, lines 3-9 (claims 13-14 and 16-19), page 12, line 3 (claim 15), page 12, lines 5-13 (claims 20-26), page 21, lines 15-19 (claims 27-28, 30 and 32), page 17, line 20 (claim 29) and page 1, line 13 (claims 31 and 33).

Consideration of new claims 12-34 is respectfully requested. It is submitted that the redrafting of the species of claims 7-9 into method claims 12-34 does not constitute a change of elected species, as the agent remains within the same species. In any case, it is submitted that the change from agent to method claims does not impose a substantial burden on the Examiner, as the substance of new claims 12-34 remains within the same species, even though the claims are now in method format. Accordingly, further examination of the method claims is respectfully requested.

Further, it is submitted that the subject matter of claims 12-34 corresponds to the species of Group I, anti-NK cell agents, which was elected in the response filed December 2, 2004 to the restriction requirement of November 17, 2004. Specifically, claim 12 recites a composition comprising at least one antibody against NK cells, wherein the at least one antibody is selected from the group consisting of antibodies identifying an NK1.1 antigen, antibodies identifying an asialo GM1 antigen, and anti-Ly 49D antibodies. Each of the at least one antibody against NK cells of claim 12 is within the elected species of Group I, i.e., anti-NK cell agents. In particular, antibodies identifying an NK1.1 antigen and antibodies identifying an asialo GM1 antigen are

antibodies that suppress NK cells, and anti-Ly 49D antibodies are antibodies that identify an antigen that activates NK cells, i.e., they are anti-NK cell agents, as discussed on page 11, lines 3-9 of the specification.

In view of the above, it is submitted that the subject matter of claims 12-34 should be fully examined.

In the Office Action, the claim for priority is acknowledged, but certified copies of the priority documents are requested.

It is submitted that the certified copies of the priority documents have already been filed as attachments to the claim for priority on October 30, 2003.

Next, in the Office Action, claims 7-9 are rejected under 35 U.S.C. 112, first paragraph, as not enabled. It is alleged in the Office Action that that the specification is enabling only for "an anti-NK1.1 antibody agent for treating dermatitis by suppressing a cell having an NK 1.1 antigen."

Reconsideration and withdrawal of the rejection is respectfully requested. Present claim 12 recites at least one antibody against NK cells selected from the group consisting of antibodies identifying an NK1.1 antigen, antibodies identifying an asialo GM1 antigen, and anti-Ly 49D antibodies. It is submitted that the present specification is enabling, not only for administration of antibodies that suppress NK cells (see Examples 2 and 3 at pages 15-17), but also for administration of antibodies against NK cells. In particular, the present inventors have disclosed and illustrated in the specification the effect of administration of antibodies identifying an antigen that activates NK cells, based on the exemplification of anti-Ly 49D antibody (see Example 5 at pages 19-20).

Further, it is submitted that the teachings of the present specification are sufficient to

enable, not only treatment, but also prevention of dermatitis and/or alopecia. Namely, the present inventors have demonstrated that administration of the agent against NK cells to mice that have been genetically modified to trigger dermatitis, starting at birth, prevents (i.e., "suppresses") any occurrence of dermatitis, whereas dermatitis is triggered in the comparative mice (see, e.g., Example 2 at page 15, lines 23-24, Example 3 at page 16, lines 25-26, and Example 5 at page 19, lines 26-27). These teachings regarding prevention and treatment are consistent with the knowledge of the processes at the source of the onset of dermatitis, as explained for example on pages 7-8 of the specification, and would be understood by the person of ordinary skill in the art as establishing effectiveness for prevention as well as treatment.

In summary, the preventive and treating effects of each of the at least one antibody against NK cells recited in present claim 12 is sufficiently disclosed, illustrated, and exemplified in the present specification, so that the present claims are enabled.

In view of the above, it is submitted that the lack of enablement rejection should be withdrawn.

Next, in the Office Action, claims 7-9 are rejected under 35 U.S.C. 102(b) as anticipated by WO 00/02923 ("Nickoloff"). It is alleged in the Office Action that Nickoloff teaches the use of antibody for CD161 antigen of NK-T cells to prevent or treat psoriasis.

Reconsideration and withdrawal of the rejection is respectfully requested. It is submitted that Nickoloff has failed to disclose an antibody against CD161 of NKT cells, because the CD1d proposed by Nickoloff is known to bind to the TCR receptor of NKT cells, and not to CD161.

In support of the above explanation, an article by Kawano et al., CD1d-Restricted and TCR-Mediated Activation of Vα14 NKT Cells by Glycosylceramides, Science Vol. 278 (1997),

pp. 1626-1629), is submitted with this paper. The Kawano article explains the known relationship between CD1d and TCR receptor (see Kawano at page 1627, right col.). This article confirms that the purported relationship between CD161 and CD1d indicated on the last line of Table II on page 12 and on Fig. 1 of Nickoloff is erroneous. In other words, Nickoloff has not shown an antibody against CD161, but has only demonstrated in its Example 3 on pages 44-45 in vitro inhibition by CD1d of peripheral cells (presumably a majority of NKT cells) isolated from a psoriatic patient.

Given these serious deficiencies of Nickoloff, a person of the art would not rely on Nickoloff as a basis for researching improvements or modifications of the method of Nickoloff. In addition, even if, arguendo, a person of ordinary skill in the art had been motivated to further study the method of Nickoloff, that person would have been aware that (i) Nickoloff has not disclosed whether a CD161 antibody would be effective against psoriasis, and (ii) TCR receptors are present on NKT cells and not NK cells. Correspondingly, that person would find no suggestion or motivation in Nickoloff to use a CD161 antibody, or other various antibodies against NK cells, in order to prevent or treat dermatitis and/or alopecia, because Nickoloff does not provide any guidance or reasonable expectation of success as to such endeavors.

In contrast, the present inventors have demonstrated that antibodies against NK cells, i.e., the antibodies as recited in present claim 12, can be used effectively to prevent or treat dermatitis and/or alopecia. In particular, the present inventors have shown that antibodies identifying an NK1.1 antigen, antibodies identifying an asialo GM1 antigen, and anti-Ly 49D antibodies can prevent or treat dermatitis and/or alopecia. The effect of these antibodies targeting NK cells is considerably more effective than the effect of targeting NKT cells as in Nickoloff. For example, Example 3 of the present specification shows that the antibody against asialo GM1 antigen, which

is present in NK cells but not NKT cells, prevented the onset of dermatitis, whereas Comparative Examples 1 and 2 (pages 16-17 and Figs. 8-9) show that antibodies against CD4 and CD8 which are present in NKT cells and not NK cells, could not prevent dermatitis. The effects of antibodies identifying an NK1.1 antigen and anti-Ly 49D antibodies to prevent or treat dermatitis are also completely unexpected from Nickoloff.

In summary, the presently claimed method of preventing or treating dermatitis and/or alopecia and its advantages are not taught or suggested in Nickoloff. Therefore, the present claims are not obvious over Nickoloff.

In view of the above, it is submitted that the rejection should be withdrawn.

In conclusion, the invention as presently claimed is patentable. It is believed that the claims are in allowable condition and a notice to that effect is earnestly requested.

In the event there is, in the Examiner's opinion, any outstanding issue and such issue may be resolved by means of a telephone interview, the Examiner is respectfully requested to contact the undersigned attorney at the telephone number listed below.

In the event this paper is not considered to be timely filed, the Applicants hereby petition for an appropriate extension of the response period. Please charge the fee for such extension and any other fees which may be required to our Deposit Account No. 50-2866.

Respectfully submitted,

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function with mAb to IFN-γ reduce the efficacy of IL-12 (*16*, *17*). Although $V_\alpha14$ NKT cells are the major source of IFN-γ (*14*), IFN-γ may not be important in the effector phase. This is because high doses of mAbs to IFN-γ do not inhibit $V_\alpha14$ NKT cell–mediated cytolysis (Fig. 3E).

We examined the potential activity of NK and T cells in vitro in the mutant mice; both NK-mediated and T cell–mediated killing functions were potent. The NK activity induced by polyinosinic-polycytidylic acid [poly(I:C)] was as potent in $J_\alpha281^{-/-}$ mice as in wild-type mice (Fig. 4A). Similarly, significant allospecific CTL activity was detected on P815 (H-2^d) and BALB/c (H-2^d) concanavalin A (ConA) blasts, but not on EL-4 (H-2^b), in $J_\alpha281^{-/-}$ mice to the same extent as in wild-type mice (Fig. 4B). Thus, NK and conventional T cells in $J_\alpha281^{-/-}$ mice are functionally active, yet not indispensable for tumor rejection upon IL-12 stimulation.

The primary effect of IL-12 on $V_\alpha14$ NKT cells is also supported by the fact that IL-12 causes an increase in the actual numbers of $V_\alpha14$ NKT cells (about a fourfold increase; 1.5×10^5 to 6×10^5) and in their cell volume (1.3 to 2.5-fold increase) (*21*). It is now clear that a reevaluation of NK and T cell functions in the absence of $V_\alpha14$ NKT cells may alter our understanding of the functions of various subsets of lymphocytes in vivo.

REFERENCES AND NOTES

1. B. J. Fowlkes *et al.*, *Nature* **329**, 251 (1987); R. C. Budd *et al.*, *J. Exp. Med.* **166**, 577 (1987).
2. Y. Makino *et al.*, *J. Exp. Med.* **177**, 1399 (1993); Y. Makino, R. Kanno, H. Koseki, M. Taniguchi, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 6516 (1996).
3. Z. K. Ballas and W. Rasmussen, *J. Immunol.* **145**, 1039 (1990); M. Sykes, *ibid.*, p. 3209; H. I. Levitsky, P. T. Golumbek, D. M. Pardoll, *ibid.* **146**, 1113 (1991); Y. Takahama, A. Kosugi, A. Singer, *ibid.*, p. 1134; S. Koyasu *et al.*, *J. Exp. Med.* **175**, 203 (1992); M. Bix, M. Coles, D. Raulet, *ibid.* **178**, 901 (1993); A. Bendelac, N. Killeen, D. R. Littman, R. H. Schwartz, *Science* **263**, 1774 (1994); T. Ohteki and H. R. MacDonald, *J. Exp. Med.* **180**, 699 (1994).
4. Y. Makino, R. Kanno, T. Ito, K. Higashino, M. Taniguchi, *Int. Immunol.* **7**, 1157 (1995).
5. K. Masuda *et al.*, *J. Immunol.* **158**, 2076 (1997).
6. K. Imai *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8708 (1986).
7. O. Lantz and A. Bendelac, *J. Exp. Med.* **180**, 1097 (1994).
8. M. Taniguchi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 11025 (1996).
9. M. Kobayashi *et al.*, *J. Exp. Med.* **170**, 827 (1989); A. S. Stern *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6808 (1990); S. F. Wolf *et al.*, *J. Immunol.* **146**, 3074 (1991); M. K. Gately *et al.*, *ibid.* **147**, 874 (1991); D. S. Schoenhaut *et al.*, *ibid.* **148**, 3433 (1992).
10. W. Hashimoto *et al.*, *J. Immunol.* **154**, 4333 (1995); M. Takahashi *et al.*, *ibid.* **156**, 2436 (1996).
11. A. Nagy, J. Rossant, R. Nagy, W. Abramow-Newerly, J. C. Roder, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8424 (1993); T. Akasaka *et al.*, *Development* **122**, 1513 (1996).
12. H. Koseki *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5248 (1990).
13. L. Kopper, T. Van Hanh, K. Lapis, *J. Cancer Res. Clin. Oncol.* **103**, 31 (1982); E. Barbera-Guillem, A. Alosno-Varona, F. Vidal-Vanaclocha, *Cancer Res.* **49**, 4003 (1989); M. Tardif, J. Coulombe, D. Soulieres, A. P. Rousseau, G. Pelletier, *Int. J. Cancer* **68**, 97 (1996).
14. T. Kawano *et al.*, *Science* **278**, 1626 (1997).
15. B. A. Teicher, G. Ara, K. Menon, R. G. Schaub, *Int. J. Cancer* **65**, 80 (1996).
16. C. L. Nastala *et al.*, *J. Immunol.* **153**, 1697 (1994); J.-P. Zou *et al.*, *Int. Immunol.* **7**, 1135 (1995).
17. M. J. Brunda *et al.*, *J. Exp. Med.* **178**, 1223 (1993).
18. J. Cui and T. Shin, data not shown.
19. T. Kataoka *et al.*, *J. Immunol.* **156**, 3678 (1996); K. Ando *et al.*, *ibid.* **158**, 5283 (1997).
20. L. K. Chen *et al.*, *Eur. J. Immunol.* **16**, 767 (1986); E. Maraskovsky, W.-F. Chen, K. Shortman, *J. Immunol.* **143**, 1210 (1989); M. J. Brunda, D. Rosenbaum, L. Stern, *Int. J. Cancer* **34**, 421 (1984); D. A. Weigent, G. J. Stanton, H. M. Johnson, *Biochem. Biophys. Res. Commun.* **111**, 525 (1983).
21. T. Shin and J. Cui, data not shown.
22. For measurement of the melanoma antigen (GM3 ganglioside), cells or tissues were homogenated. The homogenates of normal or metastatic livers (50 mg) or B16 melanoma were treated with 1 ml of extraction buffer containing 1% NP-40. Varied concentrations of melanoma cell homogenates were used to determine standard curves. Melanoma antigens in the extracts were quantified by radioimmunoassay with ^{125}I-labeled M2590 mAb to melanoma as described [S. Wakabayashi, S. Okamoto, M. Taniguchi, *Jpn. J. Cancer Res.* **75**, 427 (1984); Y. Gunji and M. Taniguchi, *ibid.* **77**, 595 (1986); G. A. Nores, T. Dohi, M. Taniguchi, S. Hakomori, *J. Immunol.* **139**, 3171 (1987)].
23. Cells (1×10^6) were first incubated with mAb to Fc γ receptors II and III (FcγRII/III) (2.4G2) to block nonspecific staining through FcγR, then the cells were incubated with fluorescein isothiocyanate–labeled anti-TCRβ (H57-597) and phycoerythrin–labeled anti-NK1.1 (PK136). Dead cells were excluded by propidium iodide staining, and 10^5 cells were analyzed by EPICS-ELITE (Coulter Electronics, Hialeah, FL) with a logarithmic amplifier as described (*5*).
24. $J_\alpha281^{+/+}$, $J_\alpha281^{-/-}$, and RAG$^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$ mice were injected with 2×10^6 B16 or FBL-3 cells in the spleen for liver metastasis, intravenously with 3×10^5 B16 or 2×10^6 LLC cells for pulmonary metastases, or subcutaneously with 2×10^6 B16 cells for subcutaneous tumor growth on day 0. Recombinant murine IL-12 (2400 U/mouse) or phosphate-buffered saline (PBS) was injected on days 3, 5, 7, and 9. On day 14, the mice were killed and either metastatic nodules counted or GM3 melanoma antigens measured by radioimmunoassay in the liver or lung as described (*22*). For subcutaneous tumor growth, injection of IL-12 or PBS was initiated on day 5, and the mice were treated five times per week. The diameters of tumors were measured every day with calipers. The sizes of tumors were expressed as the products of the longest diameter times the shortest diameter (in square millimeters).
25. Target cells were labeled with 100 μCi of sodium chloride (Amersham) per 5×10^6 cells for 1 hour. Effector cells were seeded in 96-well round-bottomed plates at indicated effector/target (E/T) ratios against 1×10^4 target cells. The release of ^{51}Cr from lysed target cells was counted on a γ-counter after a 4-hour incubation at 37°C in 5% CO_2. The percent of specific ^{51}Cr-release was calculated by the following formula: percent of specific lysis = (sample cpm − spontaneous cpm) × 100/(maximum cpm − spontaneous cpm). Spontaneous cpm was calculated from the supernatant of the target cells alone, and the maximum release was obtained by adding 1N HCl to target cells.
26. MAbs to H-2K^b (AF6-885), H-2D^b (KH-95), CD1d (1B1), $V_\beta8$ (MR5-2), NK1.1 (PK136), Ly49C (5E6), IFN-γ (R4-6A2), Fas (Jo2), and Fas ligand (K10) (Pharmingen) were used for blocking experiments.
27. S. Wakabayashi, M. Taniguchi, T. Tokuhisa, H. Tomioka, S. Okamoto, *Nature* **294**, 748 (1981).

28. We thank M. Kobayashi and S. F. Wolf, Genetic Institute Incorporated, for recombinant murine IL-12 and H. Tanabe for secretarial assistance. Supported in part by the Grant-in-Aid for Scientific Research on Priority Areas (08282103) from the Ministry of Education, Culture, and Science, Japan and the Taisho Pharmaceutical Company.

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CD1d-Restricted and TCR-Mediated Activation of $V_\alpha14$ NKT Cells by Glycosylceramides

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Natural killer T (NKT) lymphocytes express an invariant T cell antigen receptor (TCR) encoded by the $V_\alpha14$ and $J_\alpha281$ gene segments. A glycosylceramide-containing α-anomeric sugar with a longer fatty acyl chain (C_{26}) and sphingosine base (C_{18}) was identified as a ligand for this TCR. Glycosylceramide-mediated proliferative responses of $V_\alpha14$ NKT cells were abrogated by treatment with chloroquine–concanamycin A or by monoclonal antibodies against CD1d/$V_\beta8$, CD40/CD40L, or B7/CTLA-4/CD28, but not by interference with the function of a transporter-associated protein. Thus, this lymphocyte shares distinct recognition systems with either T or NK cells.

An unusual lineage of lymphocytes, $V_\alpha14$ NKT cells, are characterized by their development before thymus formation (*1*), their expression of an invariant TCR encoded by the $V_\alpha14$ and $J_\alpha281$ gene segments (*2*, *3*) mainly associated with $V_\beta8.2$ (*4*), and by the coexpression of the NK1.1 receptor, a marker of NK cells (*5*). The invariant $V_\alpha14$ TCR is essential for the development and function of $V_\alpha14$ NKT cells (*6*–*8*). Contrary to the general rule that the interaction of the TCR with the major histocompatibility complex (MHC) molecules leads to the development of T cells, $V_\alpha14$ NKT cells are selected by CD1d, a nonclassical class Ib molecule (*9*); mutant mice deficient in

CD1d lack $V_\alpha14$ NKT cells (*10*). However, a ligand for invariant $V_\alpha14$ TCR has not yet been identified. Here, we attempt to define a ligand with which to specifically activate $V_\alpha14$ NKT cells and characterize their activation mechanisms.

Because another CD1 molecule, human CD1b, presents glycolipids (*11*, *12*), and because the development and selection of $V_\alpha14$ NKT cells are independent of transporter-associated protein (TAP) essential for peptide presentation on MHC (*13*), we studied glycolipids as candidate $V_\alpha14$ TCR ligands. Synthetic glycolipids (*14*) were used to avoid effects of minor contaminants in biological samples. Moreover, we generated $V_\alpha14$ NKT mice expressing the invariant $V_\alpha14$ and $V_\beta8.2$ transgenes in a recombination activating gene (RAG)–deficient background ($RAG^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$) that only have $V_\alpha14$ NKT cells, but no T, B, or NK cells (*15*). Spleen cells from $V_\alpha14$ NKT mice were cocultured with fractionated dendritic cells (DCs) from NK-only ($RAG^{-/-}$) mice (no T, B, or $V_\alpha14$ NKT cells) pulsed with various glycosylceramides, and their proliferative responses were measured.

$V_\alpha14$ NKT cells incorporated [^{3}H]thymidine ([^{3}H]TdR) after stimulation with α-galactosylceramide (α-GalCer), whereas activation with ceramide itself or β-galactosylceramide (β-GalCer) resulted in no proliferative responses (Fig. 1, A and C). Because α-glucosylceramide (α-GlcCer) as well as α-GalCer stimulated $V_\alpha14$ NKT cells readily, the α-anomeric conformation of sugar moiety is essential. Indeed, in diglycosylated ceramides (Fig. 1, B and C), the α-anomeric configuration of the inner sugar is important. Galα1-6Galα1-1′Cer, Galα1-6Glcα1-1′Cer, Galα1-2Galα1-1′Cer, or Galβ1-3Galα1-1′Cer, whose inner sugar is either α-glucose or α-galactose despite their α- or β-anomer of the outer sugar moiety, could stimulate $V_\alpha14$ NKT cells, whereas Galβ1-4Glcβ1-1′Cer with the β-anomer inner sugar could not.

Because α-GalCer and α-GlcCer, which differ only in the configuration of the 4-hydroxyl group on the carbohydrate, showed no functional differences, the 4-hydroxyl configuration of the sugar seems not to be important. However, α-mannosyl ceramide (α-ManCer), which showed no stimulatory activity, has the 2-hydroxyl group with an axial configuration that differs from that with an equatorial bond on α-GalCer or α-GlcCer, suggesting the importance of the configuration of the 2-hydroxyl group on the sugar moiety, probably for the TCR contact site of this glycolipid (Fig. 1, A and C).

A mutant derivative lacking the 3- and 4-hydroxyl groups on the phytosphingosine of α-GalCer(3,4-deoxy α-GalCer) was not stimulatory, indicating that the 3,4-hydroxyl groups of the phytosphingosine are also important (Fig. 1, A and C). Although the 3-hydroxyl group of the sphingosine plays a crucial role in the sphingolipid-mediated fusion of Semliki Forest virus (*16*), we could not determine whether the 3- and 4-hydroxyl groups were important for the TCR contact sites or for the stabilization of glycolipid conformation.

CD1d is essential for this ligand presentation and recognition by the invariant $V_\alpha14$ TCR, because proliferative responses of $V_\alpha14$ NKT cells were abrogated by monoclonal antibody (mAb) against CD1d, $V_\beta8$, B7, CTLA-4, CD28, CD40, or CD40L but not against H-$2K^b$ or I-A^b (Fig. 2, A and B), indicating that α-GalCer–mediated stimulation of $V_\alpha14$ NKT cells is CD1d-restricted and TCR/costimulatory molecule–dependent. NKT cell hybridomas have been reported to have CD1d autoreactivity (*9*). This discrepancy may be explained by the different TCR expression of hybridomas made by the fusion of thymocytes lacking $V_\alpha14$ TCR expression on the surface (*3*). β_2-Microglobulin $(\beta_2M)^{-/-}$ DCs did not stimulate $V_\alpha14$ NKT cells, whereas $TAP^{-/-}$ DCs could (Fig. 2C), supporting nonpeptide ligand presentation by

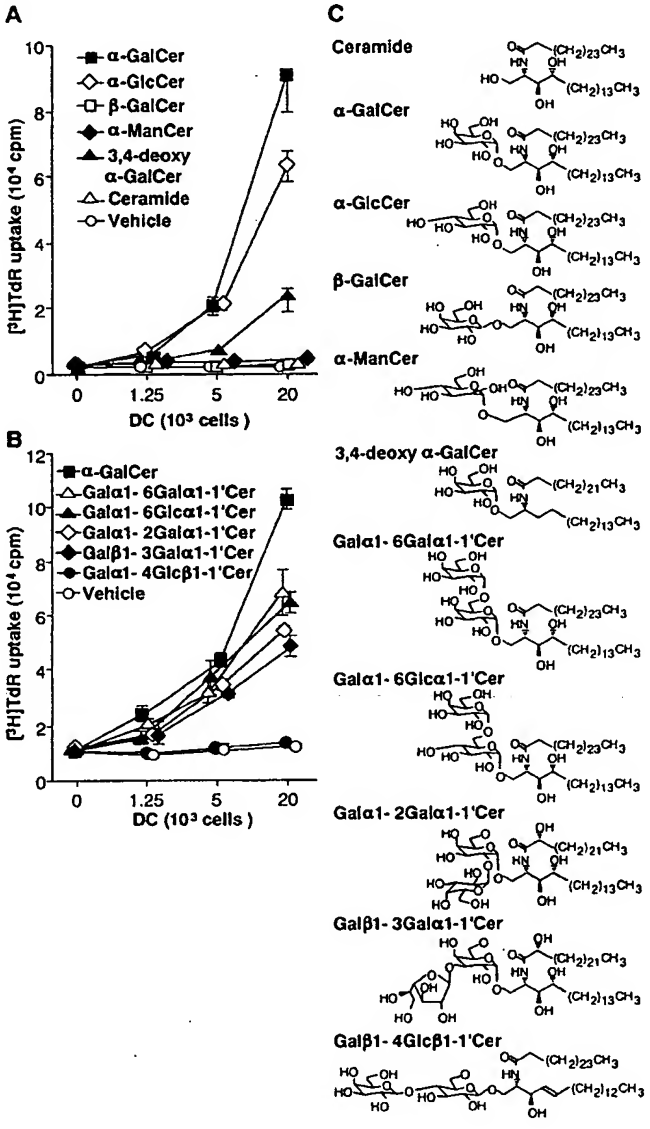

Fig. 1. Proliferative responses of $V_\alpha14$ NKT cells by glycosylceramides. Fractionated DCs prepared from $RAG^{-/-}$ mice as described by Crowley *et al.* (*30*) were pulsed with ceramide [100 ng/ml in 0.1% dimethyl sulfoxide (DMSO)–RPMI], glycosylceramides (100 ng/ml in 0.1% DMSO-RPMI), or control vehicle (0.1% DMSO-RPMI). Spleen cells (2×10^5) from $V_\alpha14$ NKT mice (*15*) were cocultured with pulsed DCs. Three days later, 0.5 μCi of [^{3}H]TdR was added for 12 hours and [^{3}H]TdR uptake was measured. (**A**) Stimulation with monoglycosylated ceramides. (**B**) Stimulation with diglycosylated ceramides. (**C**) Schematic representation of glycosylceramides. The results are expressed as mean counts per minute of three cultures ± SD.

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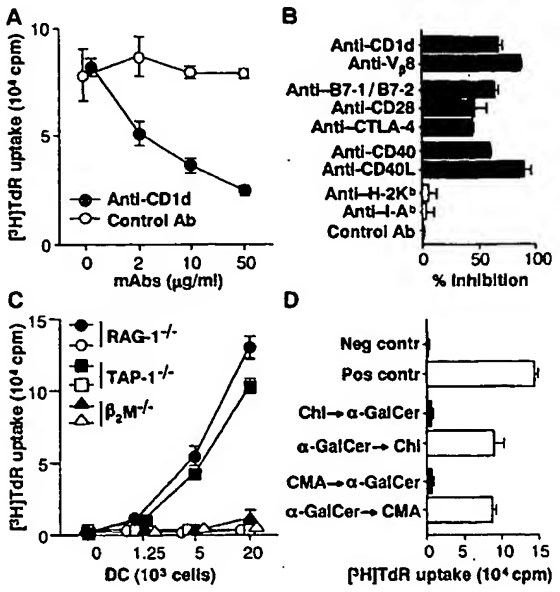

Fig. 2. Mode of recognition and activation of $V_\alpha14$ NKT cells in the induction phase. Cells were prepared as described in Fig. 1 except for the materials indicated below. **(A)** CD1d-dependent recognition. DCs (2×10^4) preincubated with anti-FcγR (50 μg/ml) (2.4G2) were reacted with anti-CD1d (1B1; Pharmingen) or its control antibody (rat immunoglobulin $G_{2b,\kappa}$). **(B)** Blocking of $V_\alpha14$ NKT cell activation. Monoclonal antibodies (50 μg/ml; Pharmingen) against CD1d (1B1), $V_\beta8$ (MR5-2), B7-1(1G10), B7-2 (PO3.1), CD28 (37.51), CTLA-4 (UC10-4F10-11), CD40 (HM40-3), CD40L (MR1), H-2K^b (AF6-88.5), and I-A^b (AF6-120.1), or control mAb, were used. The data were expressed as percent inhibition of the experimental counts per minute over the control counts per minute (81,336 ± 4050 counts per minute). **(C)** Requirement of MHC class I–like molecules but not TAP for stimulation of $V_\alpha14$ NKT cells. α-GalCer–pulsed (closed symbols) or vehicle-pulsed (open symbols) DCs prepared from $\beta_2M^{-/-}$, $TAP^{-/-}$, or $RAG^{-/-}$ mice were used (*30*). **(D)** Effects of Chl or CMA on α-GalCer presentation by DCs. Chl (100 μM; Sigma) or CMA (10 nM; Wako Pure Chemical Industries) was added to the culture of DCs (2×10^4) 1 hour before (Chl or CMA→α-GalCer) or 2 hours after (α-GalCer→ Chl or CMA) the beginning of the 4-hour pulse with α-GalCer. α-GalCer– or vehicle-pulsed DCs without treatment were used as positive (Pos contr) or negative (Neg contr) controls, respectively. The results are expressed as mean counts per minute of three cultures ± SD.

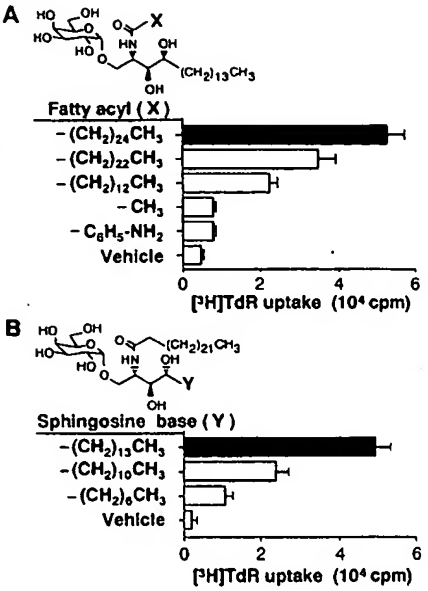

Fig. 3. Effects of lengths of fatty acyl chain and sphingosine base of α-GalCer on activation of Vα14 NKT cells. α-GalCers with different lengths of fatty acyl chains as indicated by X **(A)** and those of sphingosine base as indicated by Y **(B)** were used. The results are expressed as mean counts per minute of three cultures ± SD.

class Ib molecule. On the basis of an analogy with lipoglycan presentation (*12*), it is conceivable that chloroquine (Chl) or concanamycin A (CMA), which prevents acidification or transportation to late endosomes (17), could inhibit α-GalCer presentation by DCs. Treatment of DCs with these drugs before pulse with α-GalCer inhibited proliferation of $V_\alpha14$ NKT cells, whereas treatment after pulse with α-GalCer failed. This finding suggests a requirement for endosomal function in α-GalCer presentation (Fig. 2D).

The structural and functional relation between the lengths of fatty acyl chain and sphingosine base and activity of α-GalCer was examined (Fig. 3, A and B). The most effective lengths of fatty acyl chain and sphingosine base were C_{26} and C_{18}, respectively, whereas the short fatty acyl or short sphingosine base lost their activity, indicating the hydrophobic interaction of α-GalCer with CD1d. The α-GalCer with fatty acyl (C_{26}) and sphingosine base (C_{18}) is estimated to be about 34 Å long, with the fatty acyl chain, the sphingosine base, and the sugar moiety being 28, 17, and 8 Å long, respectively (*18*). Recent studies on the crystal structure of CD1d molecule indicate that the binding groove has two large hydrophobic pockets about 30 Å long and 10 to 15 Å wide (*19*). Therefore, the findings indicate that the α-GalCer with fatty acyl (C_{26}) and sphingosine base (C_{18}) may be suitable for binding to these two pockets of CD1d, possibly through hydrophobic interactions.

To investigate the selectivity of $V_\alpha14$ NKT cell activation with α-GalCer, we cultured spleen cells with α-GalCer from wild-type littermates and $V_\alpha14$ NKT-deficient, $V_\alpha14$ NKT, and NK-only mice whose FACS (fluorescent-activated cell sorting) profiles are shown in Fig. 4A (top). Proliferative responses were observed in $V_\alpha14$ NKT and wild-type mice, but not in the mice without $V_\alpha14$ NKT cells (NK-only mice or $V_\alpha14$ NKT-deficient mice) (Fig. 4A). In addition, $V_\alpha14$ NKT mice produced large amounts of interleukin-4 (IL-4) and interferon γ (Fig. 4B) and also killed the YAC-1 cells upon stimulation with α-GalCer, whereas $V_\alpha14$ NKT-deficient or NK-only mice did not (Fig. 4C). Thus, α-GalCer selectively activates $V_\alpha14$ NKT cells in vivo but not other lymphocytes. Vα14 NKT cells directly kill target tumor cells by an NK-like mechanisms and inhibit tumor growth and metastasis in vivo after activation with α-GalCer (*20*) or IL-12 (*8*), confirming the previous data on the protection of tumor metastasis by the treatment of tumor-bearing mice with α-GalCer (*21*).

Monogalactosylceramide is the smallest size glycosphingolipid, but β-GalCer has been detected mainly in mammals (*22*). Most mammalian normal tissues have ceramides composed of sphingosine with the 4,5-*trans* carbon-carbon double bond in the sphingosine backbone, whereas α-GalCer has phytosphingosine without this carbon double bond (*23*). Although α-GalCer has been isolated from marine sponges and has been hardly detected in normal mammalian tissues (*24*), a form of α-anomeric monoglycolipid or glycolipid with phytosphingosine has been detected in certain bacteria (*25*) and in some conditions of mammalian tissues, such as fetus (*26*), cancer cells (*27*), kidney or intestine (*28*), or in some cultured cells (*29*). An α-glycosylceramide or a natural ligand similar to this glycolipid may thus exist in restricted mammalian tissues or be expressed on cells after activation or during malignancy.

REFERENCES AND NOTES

1. Y. Makino, R. Kanno, H. Koseki, M. Taniguchi, *Proc. Natl. Acad. Sci. U.S.A.* 93, 6516 (1996).
2. K. Imai *et al.*, *ibid.* 83, 8708 (1986); H. Koseki, K. Imai, T. Ichikawa, I. Hayata, M. Taniguchi, *Int. Immunol.* 6, 557 (1989); O. Lantz and A. Bendelac, *J. Exp. Med.* 180, 1097 (1994).
3. Y. Makino, R. Kanno, T. Ito, K. Higashino, M. Taniguchi, *Int. Immunol.* 7, 1157 (1995).
4. R. C. Budd *et al.*, *J. Exp. Med.* 166, 577 (1987); B. J. Fowlkes *et al.*, *Nature* 329, 251 (1987); M. Sykes, *J. Immunol.* 145, 3209 (1990); Z. K. Ballas and W. Rasmussen, *ibid.*, p. 1039; H. I. Levitsky, P. T. Golumbek, D. M. Pardoll, *ibid.* 146, 1113 (1991); K. Masuda *et al.*, *ibid.* 158, 2076 (1997).

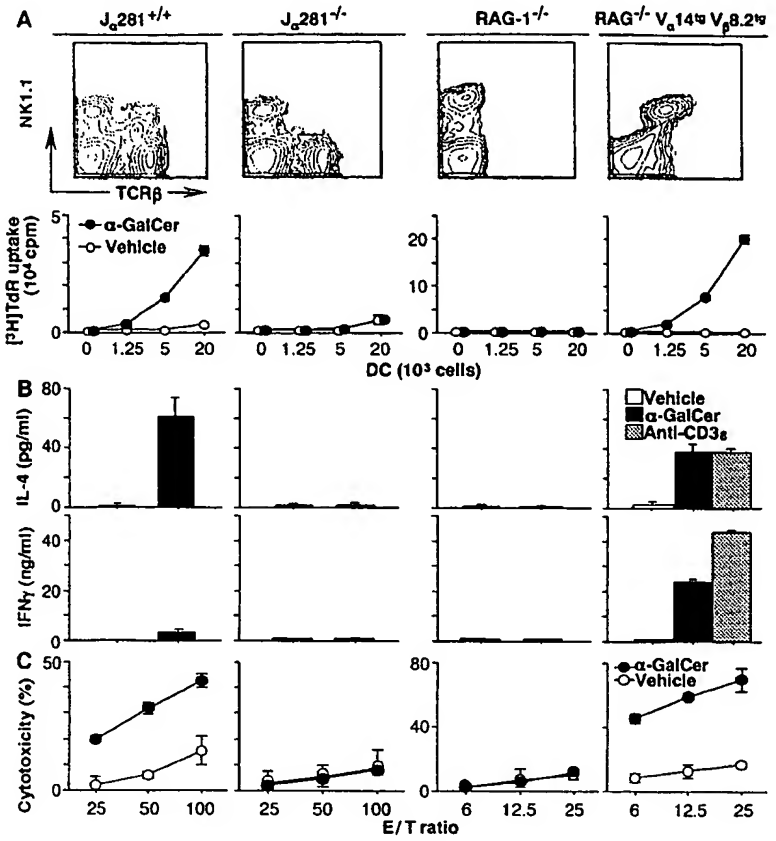

Fig. 4. Selective stimulation of $V_\alpha14$ NKT cells by α-GalCer. **(A)** FACS profiles and in vitro proliferative responses. α-GalCer–pulsed DCs were cocultured with spleen cells from wild-type ($J_\alpha281^{+/+}$) mice, $V_\alpha14$ NKT-deficient ($J_\alpha281^{-/-}$) mice, NK-only (RAG-$1^{-/-}$) mice, or $V_\alpha14$ NKT (RAG$^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$) mice as described in Fig. 1. Profiles by FACS analysis (*31*) are also shown. **(B)** Production of IL-4 and IFN-γ by α-GalCer activation. Spleen cells (2×10^5) were cultured at 37°C for 48 hours with α-GalCer (100 ng/ml), immobilized anti-CD3ε (10 μg/ml) (2C11; Pharmingen), or control vehicle. Cytokine production in the supernatants was assayed by an enzyme-linked immunosorbent assay (ELISA) kit (Endogen). **(C)** α-GalCer–mediated cytotoxicity in vitro. Mice were injected intraperitoneally with either α-GalCer (100 μg/kg in 0.025% Polysolvate 20), or vehicle–phosphate-buffered saline (PBS) (0.025% Polysolvate 20 in PBS). Twenty-four hours later, spleen cells were assayed for 4 hours on ^{51}Cr-labeled YAC-1 (H-2^{kd}) cells (1×10^4) as described (*8*). The results are expressed as mean counts per minute of three cultures ± SD.

5. L. Moretta, E. Ciccone, M. C. Mingari, R. Biassoni, A. Moretta, *Adv. Immunol.* **55**, 341 (1994); W. M. Yokoyama, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3081 (1995).
6. M. Taniguchi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 11025 (1996).
7. M. A. Mieza *et al.*, *J. Immunol.* **156**, 4035 (1996).
8. J. Cui *et al.*, *Science* **278**, 263 (1997).
9. A. Bendelac *et al.*, *Science* **268**, 863 (1995); A. Bendelac, *J. Exp. Med.* **182**, 2091 (1995).
10. S. T. Smiley, M. H. Kaplan, M. J. Grusby, *Science* **275**, 977 (1997); Y.-H. Chen, N. M. Chiu, M. Mandel, N. Wang, C.-R. Wang, *Immunity* **6**, 459 (1997); S. K. Mendiratta *et al.*, *ibid.*, p. 469.
11. E. M. Beckman *et al.*, *Nature* **372**, 691 (1994).
12. S. Porcelli, C. T. Morita, M. B. Brenner, *ibid.* **360**, 593 (1992); P. A. Sieling *et al.*, *Science* **269**, 227 (1995); M. Sugita *et al.*, *ibid.* **273**, 349 (1996); T. I. Prigozy *et al.*, *Immunity* **6**, 187 (1997).
13. Y. Adachi, H. Koseki, M. Zijlstra, M. Taniguchi, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1200 (1995); R. R. Brutkiewicz, J. R. Bennink, J. W. Yewdell, A. Bendelac, *J. Exp. Med.* **182**, 1913 (1995).
14. Synthetic glycolipids used were as follows: α-GalCer, (2*S*, 3*S*, 4*R*)-1-*O*-(α-D-galactopyranosyl)-*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol; ceramide, (2*S*, 3*S*, 4*R*)-*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol; β-GalCer, (2*S*, 3*S*, 4*R*)-1-*O*-(β-D-galactopyranosyl)- *N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol; α- GlcCer, (2*S*, 3*S*, 4*R*)-1-*O*-(α-D-glucopyranosyl)-*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol; 3,4-deoxy α-GalCer, [(2*S*)-1-*O*-(α-D-galactopyranosyl)-*N*-tetracosanoyl-2-amino-1-octadecanol]; α-ManCer, (2*S*, 3*S*, 4*R*)-2-amino-*N*-hexacosanoyl-1-*O*-(α-D-mannopyranosyl)-1,3,4-octadecanetriol; Galα1-6Galα1-1′Cer, (2*S*, 3*S*, 4*R*)-2-amino-1-*O*-(α-D-galactopyranosyl-(1-6)-α-D-galactopyranosyl)-*N*-hexacosanoyl-1,3,4-octadecanetriol; Galα1-6Glcα1-1′Cer, (2*S*, 3*S*, 4*R*)-2-amino-1-*O*-(α-D-galactopyranosyl-(1-6)-α-D-glucopyranosyl)-*N*-hexacosanoyl-1,3,4-octadecanetriol; Galα1-2Galα1-1′Cer, (2*S*, 3*S*, 4*R*)-2-amino-1-*O*-(α-D-glucopyranosyl-(1-2)-α-D-galactopyranosyl)-*N*-[(*R*)-2-hydroxytetracosanoyl]-1,3,4-octadecanetriol; Galβ1-3Galα1-1′Cer, (2*S*, 3*S*, 4*R*)-2-amino-1-*O*-(β-D-galactofuranosyl-(1-4)-α-D-galactopyranosyl)-*N*-[(*R*)-2-hydroxytetracosanoyl]-1,3,4-octadecanetriol; Galβ1-4Glcβ1-1′Cer, (2*S*, 3*S*, 4*E*)-2-amino-1-*O*-(β-D-galactopyranosyl-(1-4)-β-D-glucopyranosyl)-*N*-hexacosanoyl-4-octadecene-1,3-diol was purchased from Sigma.
15. $V_\alpha14$ NKT (RAG$^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$) mice with a C57BL/6 background were obtained by mating RAG$^{-/-}$ $V_\beta8.2^{tg}$ mice and RAG$^{-/-}$ $V_\alpha14^{tg}$ mice generated by mating RAG$^{-/-}$ mice with $V_\beta8.2^{tg}$ or $V_\alpha14^{tg}$ mice, respectively (*6*). All mice were maintained in pathogen-free animal facilities. $V_\alpha14$ NKT mice have only $V_\alpha14$ NKT cells but no other lymphocytes. Lack of NK cells in the mice was also observed in $V_\beta8^{tg}$ mice, suggesting that the transgene of $V_\beta8$ blocks NK cell development (*6*).
16. J. Corver *et al.*, *J. Virol.* **69**, 3220 (1995).
17. M. Wibo and B. Poole, *J. Cell Biol.* **63**, 430 (1974); P. E. Jensen, *J. Immunol.* **141**, 2545 (1988); J.-T. Woo, C. Shinohara, K. Sakai, K. Hasumi, A. Endo, *Eur. J. Biochem.* **207**, 383 (1992).
18. The structure of α-GalCer was generated with SYBYL software package (Tripos Associate, St. Louis, MO), and their sizes were estimated with this application.
19. Z.-H. Zeng *et al.*, *Science* **277**, 339 (1997).
20. T. Kawano *et al.*, data not shown.
21. E. Kobayashi, K. Motoki, T. Uchida, H. Fukushima, Y. Koezuka, *Oncol. Res.* **7**, 529 (1995); K. Motoki *et al.*, *Biol. Pharmacol. Bull.* **18**, 1487 (1995); M. Morita *et al.*, *J. Med. Chem.* **38**, 2176 (1995).
22. L. Svennerholm, A. Bruce, J. E. Mansson, B. M. Rynmark, M. T. Vanier, *Biochim. Biophys. Acta* **280**, 626 (1972); K. A. Karlsson, B. E. Samuelsson, G. O. Steen, *ibid.* **316**, 317 (1973); S. Ali, J. M. Smaby, R. E. Brown, *Biochemistry* **32**, 11696 (1993).
23. R. A. Laine and T. C.-Y. Hsieh, *Methods Enzymol.* **138**, 186 (1987).
24. T. Natori, Y. Koezuka, T. Higa, *Tetrahedron Lett.* **34**, 5591 (1993); V. Costantino, E. Fattorusso, A. Mangoni, *Liebigs Ann. Chem.* **1995**, 1471 (1995).
25. S. Kawasaki *et al.*, *J. Bacteriol.* **176**, 284 (1994); K. Kawahara, I. Mizuta, W. Katabami, M. Koizumi, S. Wakayama, *Biosci. Biotechnol. Biochem.* **58**, 600 (1994).
26. K. Nishimura *et al.*, *Biochim. Biophys. Acta* **796**, 269 (1984); K. Nishimura and A. Nakamura, *J. Biochem.* **98**, 1247 (1985).
27. K. Watanabe, T. Matsubara, S. Hakomori, *J. Biol. Chem.* **251**, 2385 (1976).
28. K. Okabe, R. W. Keenan, G. Schmidt, *Biochem. Biophys. Res. Commun.* **31**, 137 (1968); M. E. Breimer, *J. Lipid Res.* **16**, 189 (1975); M. W. Crossman and C. B. Hirschberg, *Biochim. Biophys Acta* **795**, 411 (1984); R. Dahiya, M. D. Brown, T. A. Brasitus, *Lipids* **21**, 107 (1986).
29. Y. Lavie, J. K. Blusztajn, M. Liscovitch, *Biochim. Biophys. Acta* **1220**, 323 (1994).
30. M. Crowley, K. Inaba, M. Witmer-Pack, R. M. Steinman, *Cell. Immunol.* **118**, 108 (1989). For preparation of fractionated DCs, spleens were digested with collagenase type III (400 U/ml; Worthington Biochemical). Single-cell suspension was loaded on a dense bovine serum albumin and centrifuged at 1500*g* for 30 min. The low-density fraction was further applied to culture dishes for 1.5 hours. Adherent cells were cultured overnight with ceramide, glycosylceramides, or control medium. The nonadherent cells in the overnight culture were used as pulsed DCs. Pulsed DCs from $\beta_2M^{-/-}$ or TAP$^{-/-}$ mice were x-rayed (30 gray) to eliminate contaminated lymphocytes before assay.
31. Cells (1×10^6) preincubated with antibody to FcγR (anti-FcγR) (2.4G2; Pharmingen) were stained with fluorescein isothiocyanate–conjugated anti-TCRβ (H57-597; Pharmingen) and biotin-conjugated anti-NK1.1 (PK136; Pharmingen) with avidin-conjugated Cy-Chrome (Pharmingen). Dead cells were gated out by propidium iodide staining, and live cells were analyzed by EPICS-XL (Coulter Electronics) with a logarithmic amplifier.

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